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Inactivation of normal function of the retinoblastoma protein (pRB) contributes to the majority of cancers including breast cancer. The broad objective of this research is to understand the impact of promotion of differentiation by pRB to its tumor suppression function. To accomplish this goal, we need to understand first function of pRB in differentiation at the molecular level. We aimed to identify proteins with which pRB interacts to promote differentiation. In screenings using Dual Bait System, we identified several cellular proteins that still interact with the pRB mutants associated with a low risk of retinoblastoma. Unlike high risk of cancer mutants, these mutants retain the ability to promote differentiation. To understand role of the identified proteins in differentiation, we started with pRB-Binding Protein 2 (RBP2) whose homolog, PLU-1, has been shown to be closely associated with the malignant phenotype in breast cancer. We propose that disregulation of RBP2 interaction with pRB in the cause of mutation may lead to cancer.

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## Table of Contents

INTRODUCTION: The retinoblastoma gene (*Rb*) is frequently mutated in many types of tumors including breast cancer [1-5]. From the emerging of cancer biology as a field, pRB has become a paradigm of tumor suppressor gene. The tumor suppression effects of pRB have been hypothesized to be due to its ability to cause cell cycle arrest. Much evidence now suggests its diverse function in differentiation. **The purpose** of my research is to identify proteins that interact with pRB to promote cellular differentiation. This would aid in understanding the contribution of pRB-mediated differentiation to tumor suppression. **The scope** of research is to search for proteins that bind specifically to pRB mutants that retain the ability to promote differentiation

BODY: To dissect pRB function in differentiation, I used selective pRB mutants recently described in our laboratory [6]. All tumor-derived pRB mutants examined to date are unable to bind transcription factor E2F-1 and are unable to repress transcription (Fig. 1). Among these, pRB mutants associated with a high risk of retinoblastoma, such as RBΔ22, encoded by a somatically altered *Rb* allele, are also unable to induce differentiation, manifested, for example, in induction of flat cells in an osteosarcoma cell line. However, certain germ-line *Rb* mutants give rise to retinoblastoma at a much lower frequency than expected for a null *Rb* allele. These partially penetrant alleles preserve, to some extent, the ability to suppress tumor formation. Our lab has addressed functional differences of the mutants associated with the low risk of cancer, for instance naturally occurring mutants RB661W, RBΔex4, and *in vitro* mutants RBΔ651 and RBΔ663. In transient transfection experiments in Saos-2 (*Rb-/-*) cells, all mutants from this category were unable to bind E2F-1 and were unable to induce a cell cycle arrest but retained the ability related to induction of differentiation such as flat cell formation and modulation activation by glucocorticoids ([6] and Fig. 2).

I used **selective pRB mutants, such as RBΔ663 and RBΔ22, in immunoprecipitation experiments and yeast two-hybrid system to identify protein interactions essential for differentiation.**

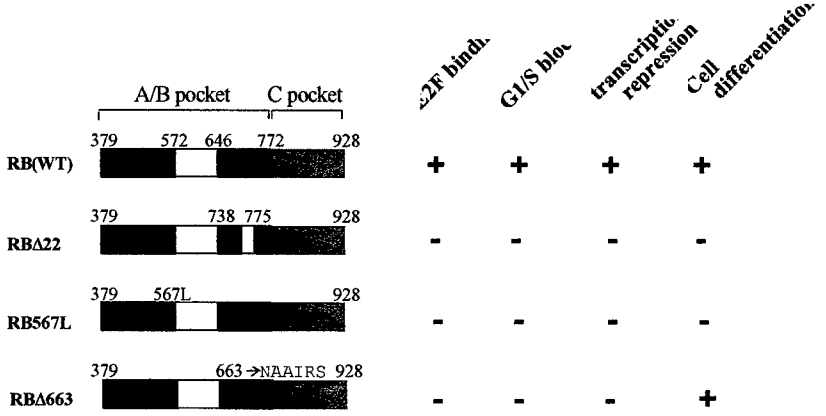


**Fig. 1. Diagram of the pRB mutants** [6; 7]. Wild-type pRB is shown from residues 379 to 928. RBΔ22 is translated from a mRNA that lacks exon 22; RB567L carries a substitution of the Leu in position 567; in RBΔ663, six nucleotides beginning at residue 663 are replaced; both mutations result in elimination protein-binding functions of the A/B pocket.

**1. Using coimmunoprecipitation assays for detection of pRB interacting proteins**

My experimental plan was to overproduce epitope-tagged versions of wild-type pRB, and selected mutants thereof, in mammalian cells using a retroviral system. **The**

**outline of performed experiments**: 1) Production of ecotropic retroviruses encoding different pRB derivatives and retroviral infection of Saos-2/EcoR (*Rb-/-*) cells; 2) Analytical immunoprecipitation of proteins from infected cells using antibodies to the epitope tag. First, I generated cell lines stably expressing ecotropic receptor (Saos-2/EcoR and U-2OS/EcoR lines, derivatives of Saos-2 and U-2OS, respectively). They can be efficiently infected with retroviruses produced by an ecotropic packaging cell line. Saos-2/EcoR cells infected with the respective pRB encoding retroviruses were analyzed for flat cell formation. As expected, the generation of flat cells, indicative of differentiation, was observed for the RB(WT) and RBΔ663, but not for the vector, RBΔ22 and RB567L. The pRB derivatives were produced as EE- or HA-tagged versions. I grew the infected Saos-2 cells in the presence of $^{35}$S methionine and performed immunoprecipitations with the anti-EE or anti-HA affinity matrix. Proteins bound to the affinity matrix were eluted with the EE (or HA) peptide and separated by SDS-PAGE gels. Bound proteins were visualized by silver staining or by using metabolic labeling of cells. Any proteins that coimmunoprecipitate with RB(WT) and RBΔ663, but not RBΔ22 and RB567L, would be of potential interest with respect to pRB's role in differentiation.

**Problems:** Using immunoprecipitation, I failed to detect a protein (call it 'X') that binds to pRB(WT) and pRBΔ663, but not to pRBΔ22 and RB567L. I'd like to mention at least several possibilities why the proteins important for differentiation are hard to isolate. One caveat is that 'X' may be unstable and/or of low abundance. I anticipated that protein 'X' may be rapidly degraded upon cell-cycle exit that is dependent on the pRB binding of MDM2 which functions as an E3 ubiquitin ligase [8]. I used a *Rb-/-* cell line defective in ubiquitin-dependent degradation to "trap" interaction of RBΔ663 with a protein 'X' which is no longer degraded, but didn't revealed any specific interactions either. pRB is bound to chromatin/nuclear matrix. Another caviat is that pRB interacting proteins are presumably low-extractable nuclear proteins. Also, epitope tag might be "hidden" from recognition by antibodies, unless exposed applying specific conditions. I have explored the influence of salt concentration and pH on binding to optimize the conditions for extraction and immunoprecipitation. Next, although I examined extracts prepared at different time points after induction of differentiation, binding of 'X' to pRB might be confined to a very narrow time interval that I failed to catch. Finally, it is possible that the pRB-'X' complex is very unstable. In other words, maybe pRB is acting 'catalytically' rather than stoichiometrically. Indeed, from my experience with RBP2 (data not shown), conditions for extraction, purification of complexes and time considerations are critical for detection of the pRB interacting proteins. Due to the failure to detect selective pRB complexes applying immunoprecipitation, I used a modified yeast two-hybrid assay as a backup strategy.

**2. Using of a modified two-hybrid system for detection of pRB interacting proteins**

The yeast two-hybrid assay is very sensitive but has the drawback of generating 'false positives'. A novel adaptation of the two-hybrid system named the dual bait system [9] allows one to minimize false positives by the simultaneous analysis of two baits in a single cell (Fig. 3). As the 1st bait, I used RB(379-928)Δ663 fused to DNA-binding

domain of LexA bacterial repressor protein. As the 2d bait, I used RB(379-928)Δ22 fused to DNA-binding domain of bacteriophage λ repressor protein cI. I transformed a human cDNA library in the yeast strain of genotype *MATα ura3 his3 trp1 clop-LYS2 lexAop-LEU2* and looked at the expression of three reporter genes indicative of interaction. **Outline of performed experiments**: 1) Subcloning of RB(379-928)Δ663 and RB(379-928)Δ22 in Dual Bait vectors; 2) Testing of the pRB fusion proteins; 3) Screening of a cDNA library for interacting proteins with the configuration of baits described in Fig. 3; 4) The DNA of the positive clones was rescued and the number of discrete genes represented among the clones was determined; 5) as a second example of Dual Bait utility, initially isolated preys were retransformed into yeast containing swapped DNA binding domain fusions. The swapped fusions eliminated a substantial number of the originally isolated clones, which were presumptively binding to a unique configuration of the original baits; 6) prey DNA was transformed in yeast carrying RB(WT) and low-penetrant mutants (Fig. 2); all of the clones, with a single exception, interacted selectively; 7) studies related to biological relevance of the identified interactions. In total, from three libraries screened, I identified six preys which interacts with pRB mutants retaining the ability to promote differentiation (Table). E7 HPV-18 oncoprotein was selected from two different fetal brain libraries. Although a legitimate RB interactor, as E7 is not normally expressed in brain, it may represent an artifact of library construction. I isolated RBP2 that was cloned originally by its interaction with pRB [10]. RBP2 is a large nuclear phosphoprotein [11]. Function of three other proteins has never been related to pRB. These are all nuclear proteins and contain Zn finger domains.

**Table. Positives identified in Dual Bait screenings with the pRB Δ663 (interacting) and Δ22 (non interacting) as the baits.**

| *Protein name* | *Description* | *Number of hits* | *Localization* | *Features* |
|---|---|---|---|---|
| E7 | human papilloma (HPV-18) oncoprotein | >60 | | |
| E2F-2 | E2F family of transcription factors | 3 | | |
| RBP2 | RB-binding Protein 2 | 1 | N | PHD |
| PLZF | Promyelocytic Leukemia Zinc Finger protein | 12 | N/C | C2H2 |
| HIRIP4 | HIRA interacting protein 4 (dnaJ-like) | 1 | N | dnaJ |
| AK000050 | NEDO human cDNA sequencing project | 1 | | C2H2 |

Apriori, except of E2F-1 we don't know which pRB interacting proteins fail to bind to the synthetic mutant RBΔ663. Binding of the E7 and adenovirus E1A

oncoprotein is known strongly interferes with cellular differentiation. Mutants that retain the ability to promote differentiation are expected to be able to bind to viral oncoproteins. I showed that RBΔ663 mutant interacts with the viral oncoproteins in both yeast two-hybrid system and in mammalian cells (Fig. 4). Cellular proteins that has been shown do not interact with RBΔ663 in mammalian cells, do not interact in yeast either.

Low-penetrant mutants are known to be unstable alleles with temperature-sensitive properties. To gain understanding of the biological relevance of interactions, I looked whether the interactions are temperature-sensitive. First, to check whether growing yeast at different temperatures causes gross changes in the pRB, including a misfolding, we compared binding to Large T antigen (Fig. 5). There were no much differences in binding to Large T antigen at different temperatures suggesting that pRB protein achieves proper folding at these temperatures. However, there was a reduced binding of low-penetrant mutants but not wild type pRB to RBP2 suggesting that their RBP2-binding activity is near the threshold level. In contrast, the binding of the RBΔex4 scored only at $24^0$C. Δex4 has biochemical properties that are distinct from other mutants. Proteins identified in our Dual Bait screen all showed temperature-sensitivity of binding to the pRB mutants: they didn't bind at $37^0$C (Fig. 6). Second, they bound to these mutants at $30^0$C when the E2F-1 binding was abrogated. Finally, there were conditions when all four are bound to the mutants. The conclusion is these mutants retain *in vivo* binding to a subset of cellular proteins when there is no binding to the E2F-1.

What might be the consequences of the loss of these proteins in human cells? I concentrated my efforts on RBP2, since its homolog, PLU-1 (Fig. 7), is specifically upregulated in the invasive and *in situ* components in primary breast cancers [12]. I applied RNAi technique to downregulate RBP2 expression in *Rb-/-* and *Rb+/+ p16-/-* (non-functional pRB) background (Saos-2 and U-2OS cells, Fig. 8). The steady-state level of RBP2 protein was determined by Western blot analysis. Dowregulation of RBP2 results in profound changes in cell morphology associated with G1 arrest and differentiation.

KEY RESEARCH ACCOMPLISHMENTS:

1. Identified six proteins that bind to low risk of cancer pRB mutants and wild-type pRB, but not high risk of cancer pRB mutants: RBP2, PLZF, HIRIP4, AK000050, E2F-2 and E7.
2. pRB mutants able to promote differentiation are unstable alleles and retain binding to all identified proteins when there is no binding to the transcription factor E2F-1.
3. Lack of RBP2 causes profound changes in cell phenotype associated with cell cycle arrest and differentiation.

REPORTABLE OUTCOMES:

1. Development of Saos-2/EcoR and U-2OS/EcoR osteosarcoma cell lines carrying stably integrated ecotropic receptor.

2. Development of reagents for Dual Bait System -
pRB derivatives; cDNA libraries transformed and propagated in *E. coli*: 1. Human Fetal Liver (B42 AD, Clontech). Livers pooled from 18-24 week old fetuses; 2. Human Fetal

Brain (B42 AD, OriGene Tech.). Frontal cortex from a 22-week old fetus; 3. Human Fetal Brain (GAL4 AD, Gibco BRL) from a 37-week female.

3. Developments of pRB, RBP2, PLZF, HIRIP4, AK000050 derivatives in mammalian expression vectors and RBP2 reagents for RNAi experiments.

CONCLUSIONS:

I determined which cellular proteins bind to wild-type pRB and low-risk of retinoblastoma mutants, but not to high risk of cancer mutants. I provided some evidence that at least one of these proteins, RBP2, affects cellular differentiation. One validation of my Dual Bait screening is that, besides cellular proteins, I identified also E7 oncoprotein. At least Large T antigen of SV-40 is known to interact with some low-risk of cancer mutants. This is consistent with my observations that these mutants interact with E7 in both mammalian cells and yeast two-hybrid system. Low-penetrant mutations have been suggested to be unstable alleles with temperature-sensitive protein-binding activities. So, temperature-sensitivity of interaction would be another validation of the screen. To the best of my knowledge, this is the first case, when cellular proteins interacting in temperature-sensitive manner with low-risk of cancer pRB mutants have been identified. Among the proteins interacting pRB mutants retaining the ability to promote differentiation, I identified RBP2. One might suggest that unstable interaction with RBP2, for example, may be a molecular basis for the variable frequency of tumor development in families with the phenotype of incomplete penetrance of retinoblastoma and breast cancers where pRB is mutated.

The nature of the identified proteins partially explains my failure to coimmunoprecipitate them from mammalian cells overproducing pRB derivatives. RBP2 is exclusively a nuclear protein. RBP2 and the other three proteins contain domains common to chromatin-bound proteins, so these pRB complexes are most probably attached to chromatin and not extractable by convenient methods.

The role of RBP2 in the development of breast cancer has never been explored. However, it has been shown that expression of its homolog, PLU-1 (Fig. 7), is upregulated in breast cancers, and the highest expression is seen in the invasive component [12]. It is likely that pRB acts at a "differentiation checkpoint" coordinating cell cycle exit and commitment to differentiation. I can imagine several models, perhaps the two simplest would be that: 1) RBP2 inhibits differentiation and is neutralized by pRB or 2) RBP2, in conjunction with pRB, promotes differentiation. These possibilities can be dissected using differentiation assays as described in [6], and applying the newly developed RNAi experiments. In this interest of space, let me indicate that trying to place RBP2 upstream or downstream of pRB will ultimately require the construction of RBP2 mutants that do or do not bind to pRB. While RBP2 represents immediate interest for a breast cancer program, three other identified proteins are necessary to be investigated as well.

As shown in my RNAi experiments, dowregulation of RBP2 in several times induces profound effects on the neoplastic phenotype of cells. This is consistent with upregulation of PLU-1 in breast cancers compared to benign lesions. These data make PLU-1 and probably RBP2 potentially amenable to pharmacological manipulation in tumors of breast and other tissues.

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## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
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1. **Benevolenskaya EV**. The *annexin X* gene cluster in *Drosophila melanogaster*. Genetika (Russian) 1994; 30 (supplement):15.

2. **Benevolenskaya EV**, Kogan GL, Balakireva MD, Filipp D, Arman IP, Gvozdev VA. Analysis of pseudogene nucleotide sequence reveals variability of rDNA genes in *Drosophila melanogaster*. Genetika (Russian translated as Russian Journal of Genetics in English) 1994; 30:280-286.

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APPENDICES:

**Figure legends**

**Figure 2. pRB constructs used for a detail study of specificity of interaction of isolated cDNA clones. A.** A diagram of full-length and large pocket pRB and p107 derivatives. They were fused to the cI DBD to be used in the Dual Bait System. **B.** Expression of pRB and p107 derivatives in yeast as revealed by cI immunoblotting.

**Figure 3. Selective pRB interactions in yeast Dual Bait System.** Interaction with two baits introduced in a single cell is monitored by expression of three reporter genes. To reveal interactions specifically retained by mutants defective in cell cycle arrest, we set up the system in a way to screen for proteins which interact with the Δ663, but not Δ22 mutant of pRB. Specificity of interaction is always a major issue in two-hybrid screenings. Introduction of the second bait, RBΔ22, provides internal control for interaction specificity.

**Figure 4. Δ663 pRB mutant that retains the ability to promote differentiation is able to bind to viral oncoproteins and some cellular proteins.**
**A.** The pRB derivative Δ663 and HA-tagged E7 were overexpressed in Saos-2 (*Rb*-/-) osteosarcoma cells and their interaction was determined by immunoprecipitation with anti-HA antibody (HA 11, Babco). The precipitated proteins were detected by immunoblotting with anti-RB antibody (XZ56). Input proteins are 10% of that used in immunoprecipitation. Δ663 like RB(WT) can be co-immunoprecipitated with E7 oncoprotein when coexpressed in Saos-2 (*Rb*-/-) osteosarcoma cells. On the contrary, Δ22 is not immunoprecipitated with E7. **B.** Likewise, E7 is strongly interacting with Δ663 in yeast Dual Bait system. *Top*, Large pocket of RB(WT), Δ663 and Δ22 were transformed in yeast as cI-fusions. Interaction between an activation domain-fused prey and bait was determined by measuring activity of the gus A reporter and reported by blue staining of clones. *Bottom*, Consistent with biochemical data, Δ663 does not bind to E2F-1 and EID-1, while WT does bind. Interestingly, in screening of cDNA library we isolated E2F-2 indicating that Δ663 retains residual interaction to E2F-2. We also isolated RBP2 which was first identified by screening a cDNA expression library with recombinant pRB protein.

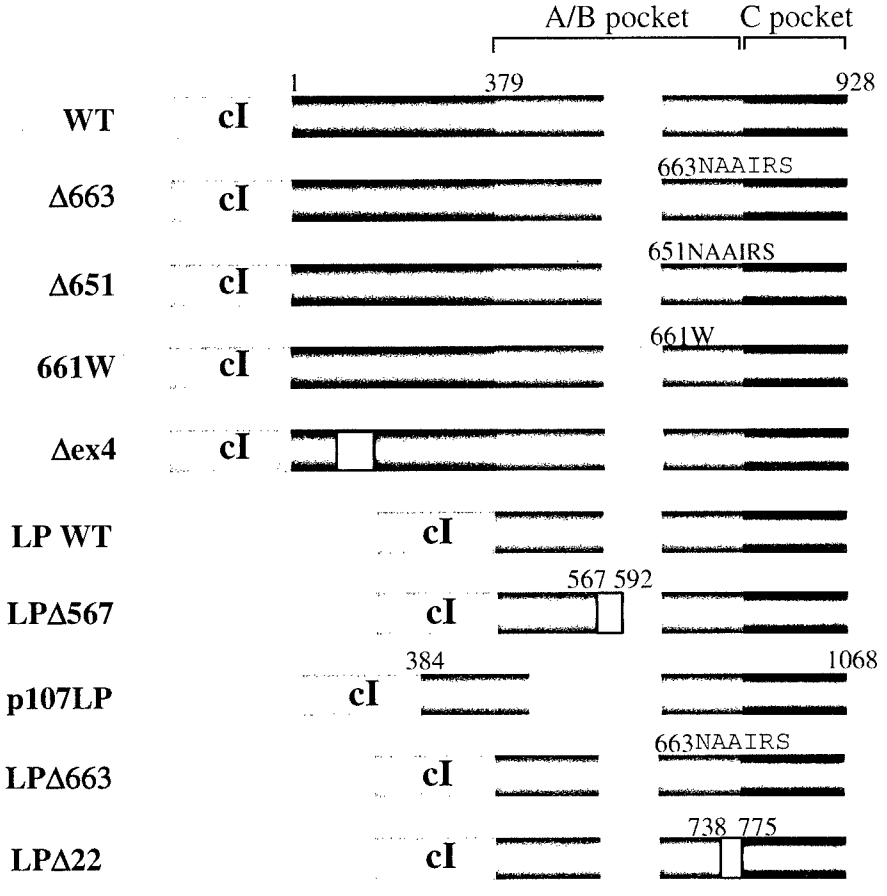
**Figure 5. RBP2-binding activity is temperature sensitive in low-penetrant pRB mutants.** Several full length RB derivatives generated as cI-fusions (Fig. 2) were transformed in yeast to be investigated for temperature-sensitivity of interaction with RBP2 and Large T antigen, as preys. The clones were replica plated on three plates and assayed for gus A reporter activity after growing cells at $23^0$C, $30^0$C or $37^0$C. Assay was performed in quadruplet.

**Figure 6. Binding to proteins identified in Dual Bait screening is temperature sensitive in pRB mutants retaining the ability to promote differentiation.** pRB derivatives (Fig. 2) were cotransformed with indicated preys in yeast and analyzed for interaction as in Figure 5. Staining was performed in quadruplet for each temperature.

**Figure 7. RBP2 is a close homolog of human protein PLU-1 upregulated in breast cancer.** Sequence alignment was done by program ALIGN in DNASTAR software.

**Figure 8. RBP2 siRNA inhibition in osteosarcoma cells.** RNAi experiments were applied to downregulate RBP2. Saos-2 and U-2OS cells were transfected with two different RBP2 siRNA duplexes or control duplexes, unrelated GL3 and scrambled. **A.** On the $5^{th}$ day after transfection, pictures of cells were taken using phase contrast microscopy. **B.** Subsequently, cytoplasmic and nuclear fractions were prepared, loaded on SDS-PAGE and immunoblotted with RBP2, pRB, or Eps15 and Lamin A as controls of fractionation. The RBP2-specific band was revealed in the nuclear, but not in the cytoplasmic fraction.

Figure 2A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p107LP
cI
663NAAIRS
LPΔ663
cI
738 775
LPΔ22
cI

Figure 2B

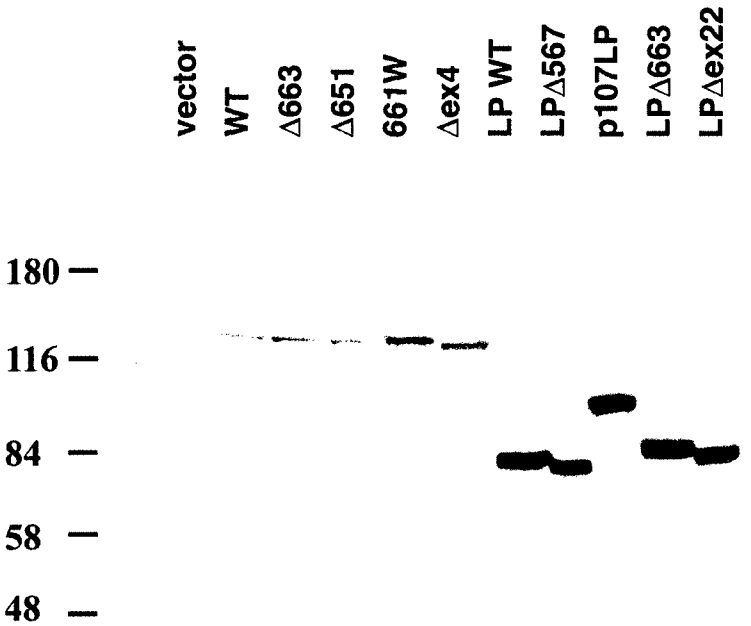
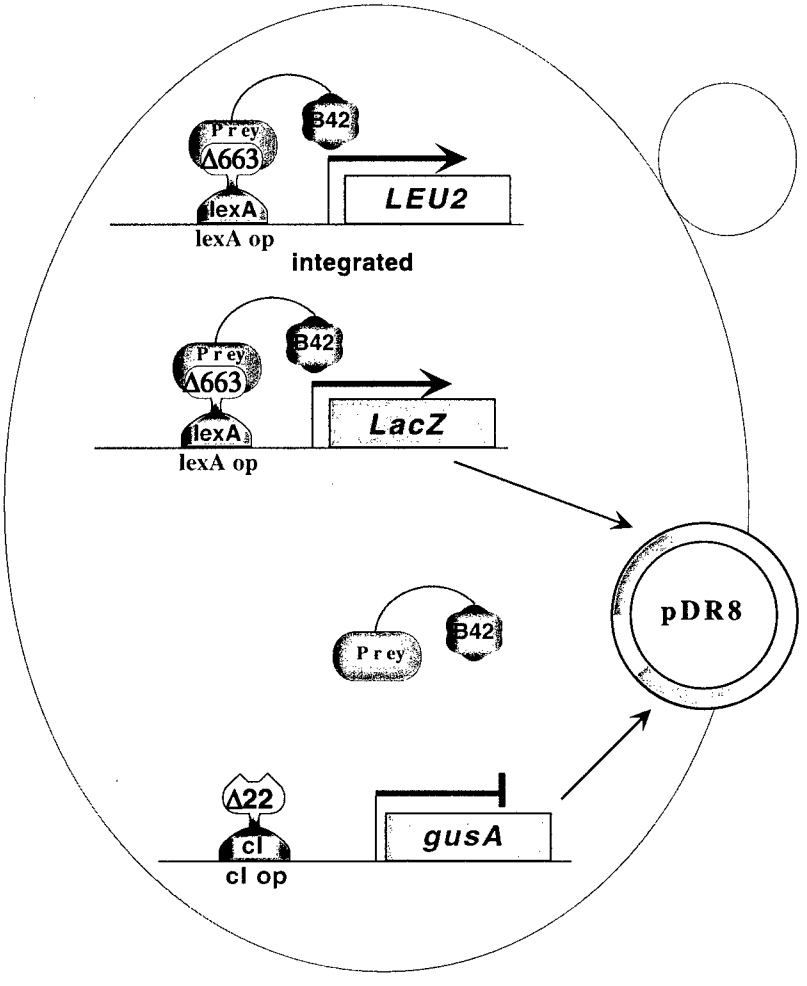

vector
WT
Δ663
Δ651
661W
Δex4
LP WT
LPΔ567
p107LP
LPΔ663
LPΔex22
180
116
84
58
48

Figure 3


Prey
Δ663
lexA
B42
LEU2
lexA op
integrated
Prey
Δ663
lexA
B42
LacZ
lexA op
pDR8
Prey
B42
Δ22
cI
gusA
cI op

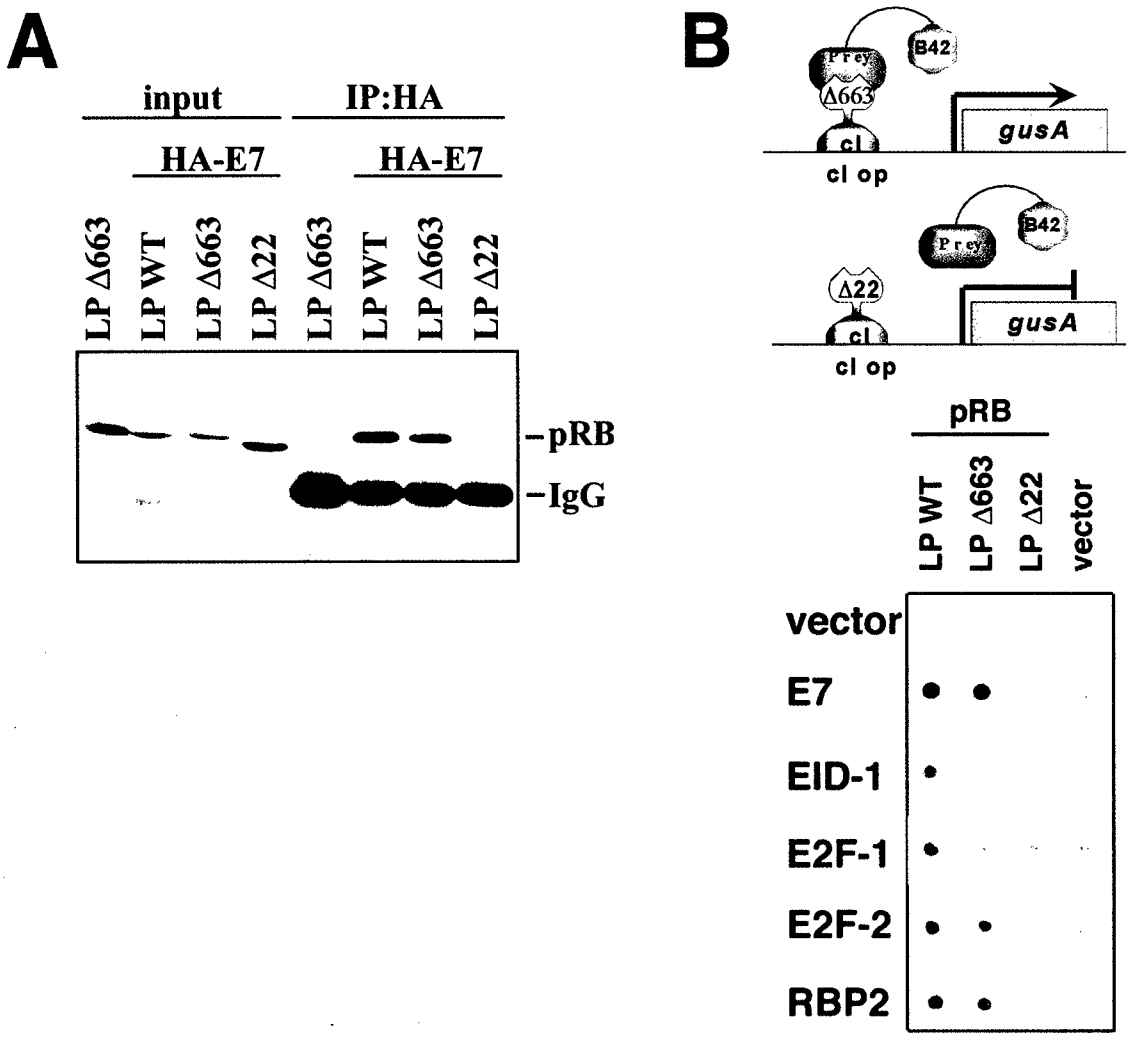

A
input
IP:HA
HA-E7
HA-E7
LP Δ663
LP WT
LP Δ663
LP Δ22
LP Δ663
LP WT
LP Δ663
LP Δ22
-pRB
-IgG
B
Prey
B42
Δ663
cI
gusA
cI op
Prey
B42
Δ22
cI
gusA
cI op
pRB
LP WT
LP Δ663
LP Δ22
vector
vector
E7
EID-1
E2F-1
E2F-2
RBP2

Figure 5

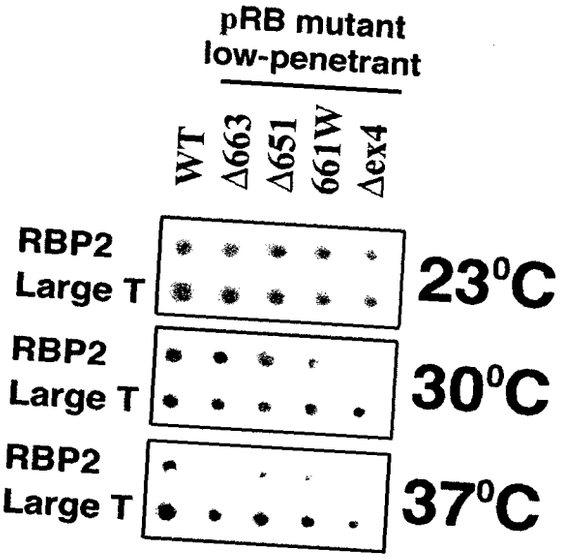

pRB mutant
low-penetrant
WT
Δ663
Δ651
661W
Δex4
RBP2
Large T
23°C
RBP2
Large T
30°C
RBP2
Large T
37°C

Figure 6

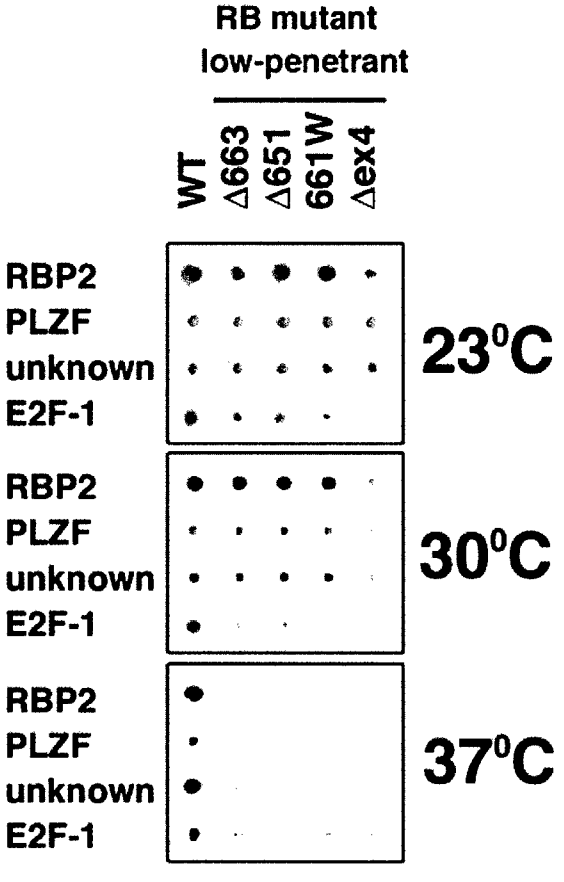

RB mutant
low-penetrant
WT
Δ663
Δ651
661W
Δex4
RBP2
PLZF
unknown
E2F-1
23°C
RBP2
PLZF
unknown
E2F-1
30°C
RBP2
PLZF
unknown
E2F-1
37°C

Figure 7

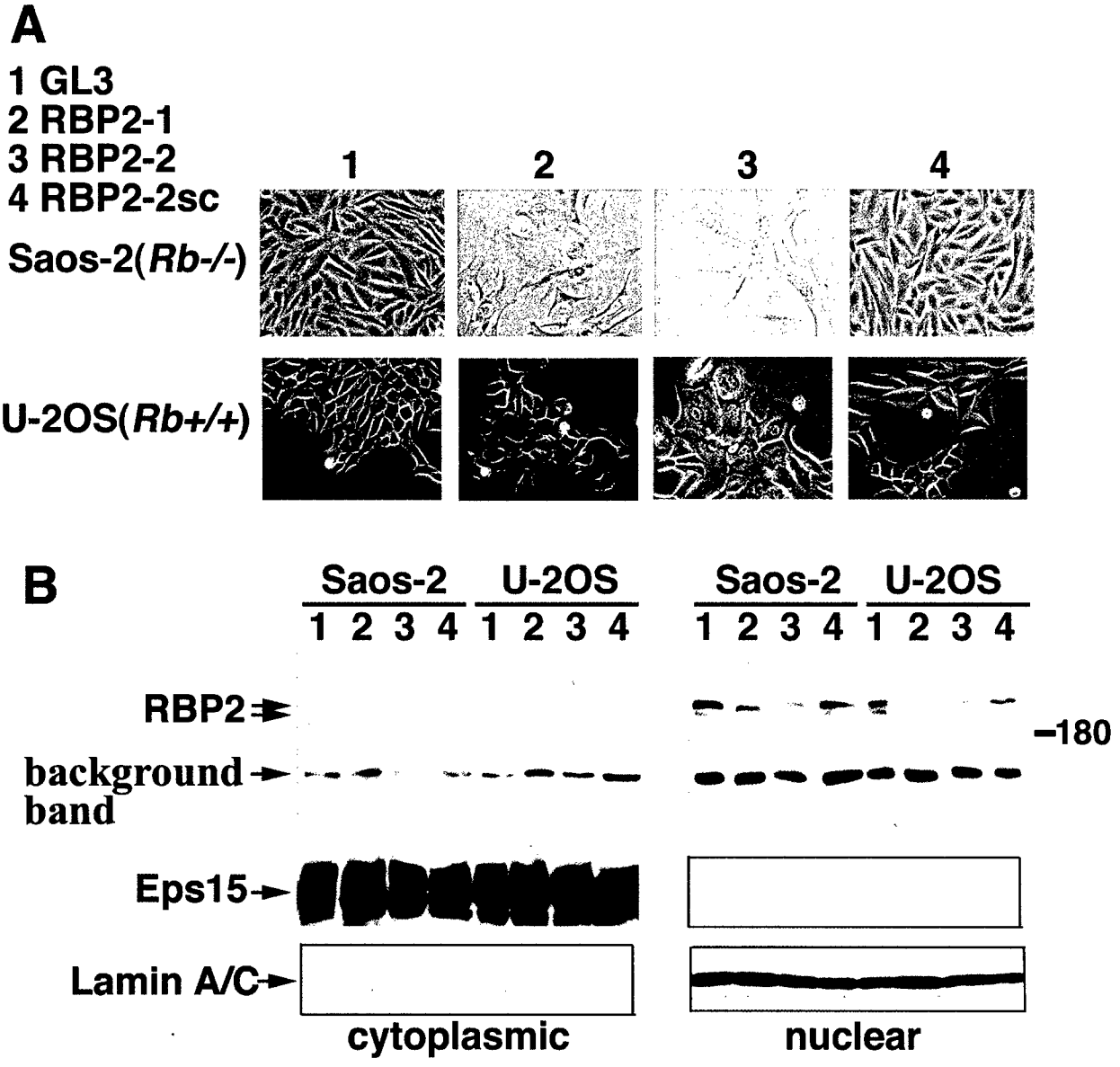

v10 v20 v30 v40 v50 v60 v70
pRBP2 VGPGGYAAEFVPPPECPVFEPSWEEFTDPLSFIGRIRPLAEKTGICKIRPPKDWQPPFACEVKSFRFTPR
G G ::EF PPPECPVFEPSWEEF.DP :FI::IRP AE TGICK RPP DWQPPFAC ::.. FTPR
pPLU-1 LGGPGPLGEFLPPPECPVFEPSWEEFADPFAFIHKIRPIAEQTGICKVRPPPDWQPPFACDGDKLHFTPR
^160 ^170 ^180 ^190 ^200 ^210 ^220
v80 v90 v100 v110 v120 v130 v140
pRBP2 VQRLNELEAMTRVRLDFLDQLAKFWELQGSTLKIPVVERKILDLYALSKIVASKGGFEMVTKEKKWSKVG
:QRLNELEA TRV:L FLDQ AK.WELQGSTLKIP:VERKILDL.:L:K VA::GGF V.K :KW:K::
pPLU-1 IQRLNELEAQTRVKLNFLDQIAKYWELQGSTLKIPHVERKILDLFQLNKLVAEEGGFAVVCKDRKWIKIA
^230 ^240 ^250 ^260 ^270 ^280 ^290
v150 v160 v170 v180 v190 v200
pRBP2 SRLGYLPGKGTGSLLKSHYERILYPYELFQSGVSLMGVQMPNL--DLKEK-----VEPEVLSTDTQTSPE
:: G.:PGK: GS: : HYERIL PY LF SG SL : Q PNL D K K :P:::S :.:::
pPLU-1 TKMGFAPGKAVGSHIRGHYERILNPYNLFLSGDSLRCLQKPNLTTDTKDKEYKPHDIPQRQSVQPSETCP
^300 ^310 ^320 ^330 ^340 ^350 ^360
v210 v220 v230 v240 v250 v260 v270
pRBP2 PGTRMNILPKRTRRVKTQSESGDVSRNTELKKLQIFGAGPK-VVGLAMGTKDKEDEVTRRRKVTNRSDAF
P::R : .:. ::K : E:: .:R L:: : :::PK .::: M:.: K: :::R: : ::::.
pPLU-1 PARRAKRMRAEAMNIKIEPEETTEARTHNLRR-RMGCPTPKCENEKEMKSSIKQEPIERKDYIVENEKE-
^370 ^380 ^390 ^400 ^410 ^420 ^430
v280 v290 v300 v310 v320 v330 v340
pRBP2 NMQMRQRKGTLSVNFVDLYVCMFCGRGNNEDKLLLCDGCDDSYHTFCLIPPLPDVPKGDWRCPKCVAEEC
: R.:K:T.:V DLYVC .CG:GN:ED:LLLCDGCDDSYHTFCLIPPL:DVPKGDWRCPKC A:EC
pPLU-1 KPKSRSKKATNAV---DLYVCLLCGSGNDEDRLLLCDGCDDSYHTFCLIPPLHDVPKGDWRCPKCLAQEC
^440 ^450 ^460 ^470 ^480 ^490
v350 v360 v370 v380 v390 v400 v410
pRBP2 SKPREAFGFEQAVREYTLQSFGEMADNFKSDYFNMPVHMVPTELVEKEFWRLVSSIEEDVIVEYGADISS
SKP EAFGFEQA:R YTL::FGEMAD:FKSDYFNMPVHMVPTELVEKEFWRLVS:IEEDV VEYGADI:S
pPLU-1 SKPQEAFGFEQAARDYTLRTFGEMADAFKSDYFNMPVHMVPTELVEKEFWRLVSTIEEDVTVEYGADIAS
^500 ^510 ^520 ^530 ^540 ^550 ^560
v420 v430 v440 v450 v460 v470 v480
pRBP2 KDFGSGFPVKDGRRKILPEEEEYALSGWNLNNMPVLEQSVLAHINVDISGMKVPWLYVGMCFSSFCWHIE
K FGSGFPV:DG: K PEEEEY:.SGWNLNNMPV EQSVLAHI :DI GMK PWLYVGMCFSSFCWHIE
pPLU-1 KEFGSGFPVRDGKIKLSPEEEEYLDSGWNLNNMPVMEQSVLAHITADICGMKLPWLYVGMCFSSFCWHIE
^570 ^580 ^590 ^600 ^610 ^620 ^630
v490 v500 v510 v520 v530 v540 v550
pRBP2 DHWSYSINYLHWGEPKIWYGVPSHAAEQLEEVMRELAPELFESQPDLLHQLVTIMNPNVLMEHGVPVYRT
DHWSYSINYLHWGEPKIWYGVP :AAEQLE VM: LAPELF:SQPDLLHQLVTIMNPN LM:H:VPVYRT
pPLU-1 DHWSYSINYLHWGEPKIWYGVPGYAAEQLENVMKKLAPELFVSQPDLLHQLVTIMNPNTLMIHEVPVYRT
^640 ^650 ^660 ^670 ^680 ^690 ^700
v560 v570 v580 v590 v600 v610 v620
pRBP2 NQCAGEFVVTFPRAYHSGFNQGYNFAEAVNFCTADWLPIGRQCVNHYRRLRRHCVFSHEELIFKMAADPE
NQCAGEFV:TFPRAYHSGFNQG.NFAEAVNFCT DWLP GRQCV:HYR.L R:CVFSH E I KMA:.:
pPLU-1 NQCAGEFVITFPRAYHSGFNQGFNFAEAVNFCTVDWLPLGRQCVEHYRLLHRYCVFSHDEMICKMASKAD
^710 ^720 ^730 ^740 ^750 ^760 ^770
v630 v640 v650 v660 v670 v680 v690
pRBP2 CLDVGLAAMVCKELTLMTEEETRLRESVVQMGVLMSEEEVFELVPDDERQCSACRTTCFLSALTCSCNPE
LDV: A: V K . M E E::LRE:V: GV SE:: FEL PDDERQC: C:TTCF SA .CSC:P:
pPLU-1 VLDVVVASTVQKDMAIMIEDEKALRETVRKLGVIDSERMDFELLPDDERQCVKCKTTCFMSAISCSCKPG
^780 ^790 ^800 ^810 ^820 ^830 ^840

Figure 7

```
          v700       v710       v720       v730       v740       v750       v760
pRBP2     RLVCLYHPTDLCPCPMQKKCLRYRYPLEDLPSLLYGVKVRAQSYDIWVSRVTEALSANFNHKKDLIELRV
          .LVCL.H:: LC CP  K  LRYRY:L DL:    : K RA:SY :W: :V EAL:A:: :KK L . ::
pPLU-1    LLVCLHHVKELCSCPPYKYKLRYRYTLDDLYPMMNALKLRAESYNEWALNVNEALEAKITKKKSLVSFKA
           ^850       ^860       ^870       ^880       ^890       ^900       ^910
          v770       v780       v790       v800       v810       v820       v830
pRBP2     MLEDAEDRKYPENDLFRKLRDAVKEAETCASVAQLLLSKKQKHRQSPDSGRTRTKLTVEELKAFVQQLFS
            E :E.:K.P NDL.R LR     AE:CASVAQ:LL:.K: :R . : G:.   LTV EL::FV.QL.:
pPLU-1    LIEESEMKKFPDNDLLRHLRLVTQDAEKCASVAQQLLNGKRQTRYRSGGGKSQNQLTVNELRQFVTQLYA
           ^920       ^930       ^940       ^950       ^960       ^970       ^980
          v840       v850       v860       v870       v880       v890       v900
pRBP2     LPCVISQARQVKNLLDDVEEFHERAQEAMMDETPDSSKLQMLIDMGSSLYVELPELPRLKQELQQARWLD
          LPCV SQ    K:LL  VE F : :Q :   ETP :::LQ L D   :  VELP:L:: : :L:QARWL
pPLU-1    LPCVLSQTPLLKDLLNRVEDFQQHSQKLLSEETPSAAELQDLLDVSFEFDVELPQLAEMRIRLEQARWLE
           ^990       ^1000      ^1010      ^1020      ^1030      ^1040      ^1050
          v910       v920       v930       v940       v950       v960       v970
pRBP2     EVRLTLSDPQQVTLDVMKKLIDSGVGLAPHHAVEKAMAELQELLTVSERWEEKAKVCLQARPRHSVASLE
          EV :.  DP.. TLD M::LID GVGLAP: AVEKAMA:LQELLTVSE W  KAK :L ARPRHS  SL
pPLU-1    EVQQACLDPSSLTLDDMRRLIDLGVGLAPYSAVEKAMARLQELLTVSEHWDDKAKSLLKARPRHSLNSLA
           ^1060      ^1070      ^1080      ^1090      ^1100      ^1110      ^1120
          v980       v990       v1000      v1010      v1020      v1030      v1040
pRBP2     SIVNEAKNIPAFLPNVLSLKEALQKAREWTAKVEAIQSGSNYAYLEQLESLSAKGRPIPVRLEALPQVES
          : V:E ::IPA.LPN:::LK : Q:AR W:::VE: Q:G . : L .L::L: :GR IPV L :LP: E:
pPLU-1    TAVKEIEEIPAYLPNGAALKDSVQRARDWLQDVEGLQAGGRVPVLDTLIELVTRGRSIPVHLNSLPRLET
           ^1130      ^1140      ^1150      ^1160      ^1170      ^1180      ^1190
          v1050      v1060      v1070      v1080      v1090      v1100      v1110
pRBP2     QVAAARAWRERTGRTFLKKNSSHTLLQVLSPRTDIGVYGSGKNRRKKVKELIEKEKEKDLDLEPLSDLEE
           VA   AW:E .::TFL :NS :.LL:VL PR.DIG :G  K.: :K KE  :::K K  .LE LSDLE:
pPLU-1    LVAEVQAWKECAVNIFLTENSPYSLLEVLCPRCDIGLLGL-KRKQRKLKEPLPNGKKKSTKLESLSDLER
           ^1200      ^1210      ^1220      ^1230      ^1240      ^1250      ^1260
          v1120      v1130      v1140      v1150      v1160      v1170      v1180
pRBP2     GLEETRDTAMVVAVFKEREQKEIEAMHSLRAANLAKMIMVDRIEEVKFCICRKTASGFMLQCELCKDWFH
          :L:E.: TA :.A ..E:: :E EA  SLR:AN.:K : .   : :K:C C K.:::.M QCELC:D.FH
pPLU-1    ALTESKETASAMATLGEARLREMEALQSLRLANEGKLLSPLQDVDIKICLCQKAPAAPMIQCELCRDAFH
           ^1270      ^1280      ^1290      ^1300      ^1310      ^1320      ^1330
          v1190      v1200      v1210      v1220      v1230      v1240      v1250
pRBP2     NSCVPLPKSSSQKKGSSWQAKEVKFLCPLCMRSRRPRLETILSLLVSLQKLPVRLPEGEALQCLTERAMS
           SCV: P  S:. . .:W       LCP:C RS::P LE:IL LL:SLQ:  VRLPEG AL:   ER  :
pPLU-1    TSCVAVP--SISQGLRIW-------LCPHCRRSEKPPLEKILPLLASLQRIRVRLPEGDALRYMIERTVN
           ^1340      ^1350           ^1360      ^1370      ^1380      ^1390
          v1260      v1270      v1280      v1290      v1300      v1310      v1320
pRBP2     WQDRARQALATDELSSALAKL--SVL-SQRMVEQAAREKTEKIISAELQKAAANPDLQGHLPSFQQSAFN
          WQ RA Q:L:.: L.  :::     L S:: :..:..:T K   :: . ::::PD.: : :S. :S:F:
pPLU-1    WQHRAQQLLSSGNLKFVQDRVGSGLLYSRWQASAGQVSDINKVSQPPGITSFSLPDDWDNRTSYLHSPFS
           ^1400      ^1410      ^1420      ^1430      ^1440      ^1450      ^1460

pRBP2     RVVSSVS
          :::S :
pPLU-1    TGRSCIP
           ^1470
```

Figure 8


A
1 GL3
2 RBP2-1
3 RBP2-2
4 RBP2-2sc
1
2
3
4
Saos-2(Rb-/-)
U-2OS(Rb+/+)
B
Saos-2
U-2OS
Saos-2
U-2OS
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
RBP2
-180
background band
Eps15
Lamin A/C
cytoplasmic
nuclear

**DEPARTMENT OF THE ARMY**
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 27 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed list of technical documents. Request the limited distribution statement assigned to the documents listed be changed to "Approved for public release; distribution unlimited." These documents should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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